AD-782 906

RESISTANCE TO VIRAL CHALLENGE IN THE DAYS IMMEDIATELY FOLLOWING VACCINATION

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Prepared for:

Army Medical Research and Development Command

May 1974

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

1

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER
AD-781906

4. TITLE (and Subtitle)
Resistance to viral challenge in the days immediately following vaccination

5. TYPE OF REPORT & PERIOD COVERED
Annual Progress Report

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
Jordi Casals-Ariet

8. CONTRACT OR GRANT NUMBER(s)
DADA17-72-C-2109

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Yale University
60 College Street
New Haven, Connecticut 06510

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

11. CONTROLLING OFFICE NAME AND ADDRESS
Department of the Army
US Army Medical Research and Development Command
Washington, DC 20314

12. REPORT DATE
1 May 1974

13. NUMBER OF PAGES
33

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report)

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

arboviruses	antibodies
vaccination	blocking of antibodies
challenge	nonspecific resistance
[illegible]-resistance	viremia

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Mice vaccinated with 17D yellow fever virus, intracerebrally challenged from 1 to 4 days later with the French viscerotropic strain of yellow fever, were substantially protected; when the challenge was with a neurotropic strain, Asibi, no protection was observed. The former strain was slow to invade and cause death with an average survival time 2 to 2½ times longer than that of Asibi strain. Challenge with a small dose of VEE virus of mice vaccinated with [illegible] vaccine revealed a moderate but significant protection 24 hours, but

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

not 7 days, after vaccination; this is another example of non-specific early resistance after vaccination.

Cytoxan given repeatedly at the time of vaccination and challenge to inhibit early non-specific resistance after vaccination. It would seem that with an adequate model it can be shown that this early resistance is independent of the immune system.

Resistance following vaccination of guinea pigs with EEE virus is apparent in animals challenged 3 or more days later; with an earlier challenge resistance seems to exist but the small numbers of animals permitted no statistically significant estimates.

Titration of antibodies following challenge of guinea pigs with arboviruses has been used to determine development of early--and late--resistance following vaccination. The vaccine, when effective, blocks the production of antibodies; observations have been made with EEE and JE viruses.

SUMMARY

Mice vaccinated with 17D yellow fever virus, intracerebrally challenged from 1 to 4 days later with the French viscerotropic strain of yellow fever, were substantially protected; when the challenge was with a neurotropic strain, Asibi, no protection was observed. The former strain was slow to invade and cause death with an average survival time 2 to 2 1/2 times longer than that of Asibi strain. Challenge with a small dose of VEE virus of mice vaccinated with JE vaccine revealed a moderate but significant protection 24 hours, but not 7 days, after vaccination; this is another example of non-specific early resistance after vaccination.

Cytoxan given repeatedly at the time of vaccination and challenge failed to inhibit early non-specific resistance after vaccination. It would seem that with an adequate model it can be shown that this early resistance is independent of the immune system.

Resistance following vaccination of guinea pigs with EEE virus is apparent in animals challenged 3 or more days later; with an earlier challenge resistance seems to exist but the small numbers of animals permitted no statistically significant estimates.

Titration of antibodies following challenge of guinea pigs with arboviruses has been used to determine development of early--and late--resistance following vaccination. The vaccine, when effective, blocks the production of antibodies; observations have been made with EEE and JE viruses.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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BODY OF REPORT

I. Statement of problem

Vaccination against virus infections of man is an accepted preventive procedure when the virus in question is universally prevalent in time and space. Arbovirus infections are, in general, geographically restricted; in addition, they are numerous and may present antigenic varieties. Under the circumstances, even available vaccines or others that may be developed in the future are to be recommended only when travelling into an endemic zone or in the face of outbreaks in heretofore free areas. In such situations, for a rational use of the vaccine it would be helpful to have information concerning the time needed, in days or hours, for substantial effective immunity to develop following vaccination.

II. Background

Over 350 arbovirus serotypes are currently recognized of which only a small number, 20 to 25, are known to cause disease of man to an extent or severity which warrant efforts to develop and apply preventive measures, among which is vaccination. Among these viruses, in addition to yellow fever for which an effective vaccine is in use are: the equine encephalitis viruses--EEE, WEE, and Venezuelan (VEE); St. Louis (SLE), Russian tick-borne (RSSE), Japanese (JE), a vaccine for which is widely used in Japan, Kyasanur Forest disease (KFD) and the four dengue serotypes, either as classical dengue or in the forms of dengue hemorrhagic fever and dengue shock syndrome. It is conceivable that infection by Crimean hemorrhagic fever-Congo (CHF-C) virus may, in the future, be added to the list in view of its wide area of distribution and the severity of the disease that it can cause in certain countries.

Simultaneous or sequential infection of an individual by antigenically closely related viruses, or their antigens, may be harmful, as has been suggested to explain the dengue shock syndrome (1,2). Recent experimental work in this laboratory (3) shows that with Lagos bat virus there is a delay in the evolution of illness in immunosuppressed mice compared with that in immuno-competent controls; and that the administration of hyperimmune serum to the infected immunosuppressed animals results in an acceleration of their illness. It would seem therefore that unless a vaccine gives effective substantial protection when administered almost simultaneously with the actual infection, the use of a vaccine may be inadvisable.

It has been shown in this laboratory (see first year's annual progress report, and 4) that mice vaccinated once with formalin-

inactivated suspensions of mouse brain tissue infected with EEE, VEE, JE, and Banzi viruses were protected against a challenge given less than 24 hours after vaccination. The protection had two phases, the first lasting from 24 to 48 hours after vaccination which was nonspecific, i.e., appeared between antigenically unrelated viruses; this was followed by a specific phase demonstrable only against the homologous virus challenge (possibly also against related viruses; this was not investigated). There was evidence that the early phase was associated with an interferon-like activity which was manifested by the serum of vaccinated mice. An immunosuppressant, Cytoxan (cyclophosphamide), was effective, as anticipated, in suppressing resistance to a challenge in the course of the specific phase; its effect on the early, nonspecific phase had been unpredictable.

III. Approach

The principal goal of the work described in this report has been to determine whether development of early resistance following vaccination could be observed in a species other than the mouse; the guinea pig was chosen.

The susceptibility of guinea pigs to arboviruses important as cause of disease of man yet relatively safe to work with in the laboratory is, if anything, lower than that of mice; it would be necessary to work with large numbers of animals which is an impossibility imposed by cost and the available housing. It was decided, after preliminary tests, to try to develop criteria other than death or survival as a measure of resistance and susceptibility; viremia and, particularly, antibody development have been used.

In continuing our studies with mice as experimental animals, two challenge viruses were added with which little or no information is available: VEE and yellow fever. All the laboratory personnel have been vaccinated against these viruses and are known to be immune.

Finally, additional studies have been conducted on the effect of Cytoxan on the early phase of resistance following vaccination.

IV. Materials and Methods

Animals. Mice derived from the Charles River CD(R)-1 strain were used, random bred in a barrier colony maintained in this laboratory. In experiments in which VEE was the challenge virus were used mice from 80 to 90 days old; for test using other challenge viruses the mice were between 30 and 40 days old. Mice from 1 to 4 days old were employed for preparation of vaccines, titration of neutralizing (N) antibodies and preparation of virus stocks and complement-fixing (CF) antigens.

Guinea pigs were purchased from commercial dealers or from the laboratory animal supply facility maintained at Yale Medical School The guinea pigs were albino, Hartley strain, either males (average weight 500 gr.) or females (350-400 gr.).

Viruses. The following viruses and strains have been employed: EEE, Alabama strain 09-28-0930, 6th to 10th mouse passage; VEE TC 80, attenuated strain developed by WRAIR by tissue culture passage to use as vaccine, was employed at mouse passage one or two; VEE, Tr-donkey 1945 strain, mouse passage 10-12; JE, Nakayama strain, mouse passage unknown but probably between 100 and 200; Banzi, H336 strain, mouse passage 6-9; yellow fever, 17D strain, vaccine supplied by Merrell-National Laboratories, mouse passage 1 or 2; and yellow fever, French viscerotropic strain, a strain which has been maintained by monkey-to-monkey injection of infected monkey serum with never before and intracerebral passage in mice; for the current tests, a mouse passage 1 or 2 has been used.

Vaccines. Yellow fever 17D vaccine was used fully active, all other vaccines were inactivated; 0.5 percent formalin was added to a 10 percent suspension of infected mouse brain tissue in physiological saline. The suspension was held at 4°C for a minimum of 7 days before use, when safety testing indicated that no virus was present. The vaccine was used for periods up to 2 months and was always kept at 4°C. In our previous experience, the vaccines were stored at -60°C. This procedure has been discontinued as it has resulted in, at least, one failure to immunize animals; it was observed that on thawing some of the vaccines kept for several months there appeared heavy flucculation or clumping which may have resulted in denaturation of the antigen.

The titers of the virus in the vaccines (IC LD_{50}/ml) prior to inactivation, determined by intracerebral (ic) titration in newborn mice were: EEE, 10^{8}-10^{-9}; VEE (TC80), 10^{9}; JE, 10^{8}; and yellow fever (17D), $10^{6.7}$.

Vaccination. Mice were vaccinated by the intraperitoneal (ip) route of inoculation: 0.2 ml of vaccine was given, either undiluted or at dilution 1:10 in physiological saline. Guinea pigs were given the vaccine by the intramuscular (im) route; 0.5 ml of vaccine was injected into each hind leg for a total of 1 ml of vaccine, undiluted or at dilution 1:10; the amount of vaccine was divided and given in two sites to minimize leakage outside the muscle tissue. When in an experiment there were groups of animals of different time intervals between vaccination and challenge, the vaccine was given on different days and all groups, including controls, were challenged on the same day.

Challenge. Mice were challenged either by the subcutaneous (sc) or ic routes of inoculation. For the sc challenge mice were inoculated

0.2 or 0.3 ml of a virus suspension in the inguinal region; ether anesthesia was used to prevent struggling and to expedite the procedure; the ic challenge consisted of 0.03 ml of a suspension, also under deep anesthesia.

Guinea pigs were challenged by the ip route, by injecting 1 ml of the given virus suspension; this route was used in preference to the sc route--a more natural route--because exploratory tests with EEE virus showed that the former route resulted in a higher mortality over a range of dilutions, from 10^{-2} to 10^{-6}.

Virus suspensions employed in a challenge were, as a rule, titrated ic in newborn mice.

Administration of Cytoxan. This drug (Mead and Johnson) was resuspended, as prescribed, in sterile water at the time when used; the resulting solution contained 20 mg of cyclophosphamide in 1 ml. The drug was given once or repeatedly, as planned, by the ip route in an amount of 150 mg/kg body weight each time; this amount was contained in a volume of either 0.2 ml or 0.25 ml, depending on the age, sex, or weight of the animals and equaled 4 or 5 mg of Cytoxan to a mouse for each injection.

Antibodies. Complement-fixing (CF) antibodies were determined by the method routinely used in this laboratory (5), consisting essentially on a semi-microtest with a total reagent volume of 0.15 ml in each well, or 6 drops, with incubation of the first phase for 18 hours at 4°C. The sera were inactivated at 60°C for 20 minutes and used in increasing two-fold dilutions beginning at 1:4 or 1:8. One or two dilutions of antigen were used, containing 4 and 16 units of antigen, respectively; as the titers given by the sera against the two doses of antigen were very similar, eventually only one dose was used, 16 units. A control antigen, i.e., an antigen prepared with an unrelated virus was used in all tests.

Neutralizing (N) antibodies were determined by the ic route of inoculation in newborn mice; undiluted serum and virus in increasing ten-fold dilutions were incubated for 2 hr at 37°C prior to inoculation.

Interpretation of challenge results. In experiments in which death or survival was the basis for evaluation of the effectiveness of a vaccine or treatment, the chi square test in 2 X 2 tables with Yates' correction for small numbers was used.

V. Results

Effect of Cytoxan on early-phase resistance. On the assumption that inconclusive results obtained earlier may have been occasioned by an excessive challenge dose, the dosage of virus in the current experiments

was adjusted to yield between 3 and 10 LD_{50}.

Test 1. Three hundred and twenty (320) mice were divided in four groups, as shown in Table 1. Groups 1 and 3, each consisting of 64 mice were given JE vaccine in the morning, 0.2 ml ip, while groups 2 and 4 each consisting of 96 mice, were left unvaccinated. Six hours later, groups 1 and 2 were given 5 mg Cytoxan ip, but not groups 3 and 4. Twenty-four hours after vaccination all mice were challenged with EEE virus, 0.2 ml sc, in the dilutions and with the result shown in Table 1. As the table indicates the administration of Cytoxan did not affect in the least the non-specific resistance induced by the vaccine against a challenge given 24 hours later. With the amount of Cytoxan given, furthermore, there was no discernable change in the susceptibility of the unvaccinated mice to EEE virus.

Test 2. On the assumption that if Cytoxan had a depressing effect on early-phase non-specific resistance it might be more easily uncovered by increasing the dosage, the experiment outlined in Table 2 was carried out.

Mice receiving Cytoxan were given 2 injections of the drug, one each on days 1 and 3; mice receiving no Cytoxan were injected, instead, physiological saline on those days. EEE vaccine was given to the corresponding groups, 1 and 3 on day 1, after the injection of Cytoxan or saline. On day 2, i.e., 24 hours after vaccination, all mice were challenged sc with Banzi virus at dilution 10^{-8}, which was anticipated to contain between 1 and 5 IP LD_{50}, with the result shown in Table 2.

The results in Table 2 confirm once more our previous observations of the development of a non-specific resistance induced by EEE vaccine against Banzi virus; even though the difference between vaccinated and controls, groups 3 and 4, is moderate considering the amount of virus in the challenge, 3 LD_{50}, the chi square value is significant, 5.7. The administration of Cytoxan did not abolish the resistance induced by the vaccine; Cytoxan increased somewhat the susceptibility of the control, unvaccinated mice, so that the inoculated challenge contained now 11 LD_{50}; and against this challenge, the Cytoxan treated, vaccinated mice had a mortality 21 of 32 which differed from that seen in the controls, 30 of 32 by a difference which has a chi square value of 6.1.

Test 3. In a test described in last year's report, repeated injections of Cytoxan over a 6-day period gave inconclusive results on the early phase resistance. In a current test, a large amount of the drug was injected to two groups of mice (see Table 3), no. 1 and 2; the drug in amounts of 4 mg per mouse--average body weight, 25 gr--was given on days 1, 3, 4, 5 and 8, while two other groups, no. 3 and 4, received no inoculum. This amount of Cytoxan was toxic resulting in signs of illness followed by death in 9 of 64 mice; the Cytoxan associated deaths occurred on days 8 and 9. Vaccinated mice, groups 1 and 3, received JE vaccine on day 3, and all mice were challenged with JE virus on day 4, or 24 hours after vaccination; the result of the challenge is shown in Table 3.

Cytoxan-inoculated mice which died as a result of the action of the drug have been eliminated from the figures given in the table. There was no diminished early resistance in the mice given Cytoxan; in fact since the susceptibility of the mice to the virus increased through the action of the drug, the early resistance induced by vaccination was if anything increased. This is in sharp contrast with the known effect of Cytoxan on the immune response following vaccination with a challenge with the homologous virus given 7 days later, as was described in last year's report.

Early resistance against yellow fever and VEE viruses in mice. Yellow fever and VEE are infections of considerable importance in relation to this project. Unfortunately it has not been possible to develop with yellow fever given to mice a model similar to that used with other arboviruses; yellow fever virus fails to kill mice after peripheral inoculation of even the largest amounts, 10^6 or 10^7 IC LD_{50}. Attempts have been made to detect, shortly after vaccination, indications of resistance against an ic challenge, even though this was considered to be an entirely artificial model.

The results of two experiments in which the Asibi strain was used for the challenge are given in Table 4. The mice had been vaccinated once with live 17D virus, approximately 10^6 LD_{50}, given ip and challenged by ic route on the indicated days. The LD_{50} of the virus in the control mice was $10^{-8.2}$ in test no. 1 and $10^{-7.8}$ in test no. 2. As the table shows, not the slightest protection was observed against $10^{2.2}$ or $10^{3.2}$ LD_{50} of virus, either 1 or 7 days after vaccination. With a smaller dose of challenge virus, test no. 2, the mice vaccinated 7 days before challenge revealed a significant degree of protection against 6 LD_{50} (dilution 10^{-7}); the chi square between vaccinated and controls (24/29, 38/40) was 11.1; there may also have been some slight protection in the next higher dilution, 10^{-8}, with a chi square value of 4.8. No protection whatsoever was observed when mice were challenged 24 hours following vaccination.

Since tests with Asibi strain as challenge showed little promise, tests were next done with the French viscerotropic strain; since it had never before been propagated in mice the assumption was made that it may be less invasive for this host than the Asibi neurotropic strain and offer, therefore, a better chance of survival to vaccinated mice. The difference in activity in mice of the two strains following ic inoculation is shown in Table 5, in which have been summarized the results obtained in several experiments. It can easily be calculated from the data in the table that the LD_{50} of the Asibi strain was $10^{-8.0}$/0.3 ml; that of the French viscerotropic strain $10^{-6.2}$/0.3 ml. It is of interest to note that the average survival time (AST) of the mice that succombed was at all dosage levels twice as long with the French viscerotropic strain than with the Asibi.

The result of a challenge test with the French viscerotropic strain is given in Table 6. Groups of mice were vaccinated once with live 17-D virus at 4 consecutive days before challenge and another unvaccinated group served as control. From the outcome in the controls it can be calculated that the LD_{50} was $10^{-6.2}$; all vaccinated mice,

including those vaccinated 24 hours before challenge, were substantially protected against 1.1 LD_{50} (dilution 10^{-6}) and 11 LD_{50} (dilution 10^{-5}).

Vaccination and challenge with VEE virus. Groups of mice were vaccinated once with either VEE (TC80) or JE (Nakayama) formalin inactivated vaccines on days 1 and 7 before challenge with VEE (Tr donkey) virus. The LD_{50} of a stock of the latter after sc inoculation in 80- to 90-day-old mice had been previously determined as being $10^{-9.6}$. In the present test, dilution of virus 10^{-9} was used as challenge; the result is shown in Table 7.

The challenge dilution contained 2.5 SC LD_{50}; all mice vaccinated with VEE (TC80) virus were solidly protected 1 and 7 days after vaccination. It is also shown in the table that the JE vaccine had a distinct protective effect when given 24 hours before challenge (8/32 vaccinated compared with 26/32 controls, died, chi square = 18.0) but not when administered 7 days before challenge.

<u>Early resistance against EEE virus in guinea pigs, following vaccination.</u> Intraperitoneal inoculation of guinea pigs with EEE virus results in fatal infection in only a fraction of the animals; in limited observations, it appeared as though the lethality was the same over a range of virus dilutions, from 10^{-2} to 10^{-6}. Although a similar situation occurs in adult mice inoculated subcutaneously, it is possible by using relatively large numbers of them to evaluate the significance of results on a statistical basis; the use of such large numbers is not realistic with guinea pigs. Daily viremia titrations as an indication of infection with EEE could, probably, be used; it was not, however, a practical procedure with our guinea pigs experiments. Attempts have been made to determine whether development of antibodies in survivors following challenge of vaccinated and control animals could be utilized as a criterion of resistance or susceptibility to the challenge.

Guinea pigs were challenge from 1 to 7 days after a single vaccination, consisting of 2 im injections, 0.5 ml each, given at the same time. The ip challenge, 1 ml of dilution 10^{-5} of infected mouse brain, contained on the average $10^{5.7}$ $ICLD_{50}$, when titrated in newborn mice. In all except one test, homologous EEE vaccine was used; in a test, heterologous JE vaccine was employed. Guinea pigs that died following challenge generally did so between days 5 and 8 with little difference in AST amongst the various groups. The results of a number of experiments are presented in Tables 8, 9, and 10.

The overall mortality of unvaccinated controls was 68.5 percent (Table 10); in separate experiments it was (Table 8) from 53 to 80 percent. Administration of JE vaccine 24 hours before challenge did not reduce the mortality appreciable, the difference between 61.5 and 68.5 percent being insignificant; even if the comparison is made (Table 8) between the mortalities in JE vaccinated guinea pigs and in the controls of the test, number 4, in which the JE vaccinated animals were included, the difference from 61.5 to 80 percent, although appreciable, is not significant with

the numbers of animals in the groups. Guinea pigs vaccinated 7 days before challenge were solidly immune. Vaccination 3 or 4 days before challenge (Table 8) resulted in significant protection. even with the small numbers of animals used. Vaccination 1 or 2 days before challenge while giving no statistically significant protection in the separate individual tests had a definite positive trend; the mortality of the combined 1- and 2-day old pre-challenge vaccinated groups was 31.6 percents while that of the total control group was 68.5 percent.

A number of guinea pigs that survived challenge were bled between 18 and 24 days later; also bled at the same time were vaccinated but unchallenged animals. The sera were tested for CF and N antibodies with the result shown in Table 9.

The results of the CF tests are particularly interesting. Following challenge of unvaccinated animals, 15 survivors tested had generally developed antibodies at high titers, 1:64 and higher; in contrast, vaccinated unchallenged guinea pigs had either no detectable antibodies or only at low titer, 1:8. Vaccinated guinea pigs challenged 7 days after vaccination responded in a manner similar to that of the vaccinated unchallenged animals: they either had no detectable antibodies, 12 or 15, or, with only one exception, the titers were low. Guinea pigs vaccinated with EEE vaccine and challenged from 1 to 4 days later fell in an intermediate area; few were negative, a number developed elevated titers, but the majority presented only moderate titers. Interestingly, guinea pigs given the heterologous vaccine, JE, 24 hours before challenge also fell in the intermediate area.

The results of the N test, in terms of neutralization indices of undiluted sera, are less sharply defined than those of the CF test when comparing the unvaccinated controls, vaccinated and challenged 7 days later and unchallenged vaccinated animals; the trend, however, parallels the results of the CF test. Conceivably, the use of serum dilutions in the N test would be a more accurate picture.

The results of all the challenge, CF and N tests have been assembled and summarized in Table 10. Owing to the fact that some of the similar categories consisted of small numbers, they have been combined in the table. Furthermore, and in order to underscore certain trends, the results of the CF test have been distributed in three classes: negative or less than 1:8 titer; intermediate titers, 1:8, 1:16, 1:32; and high titers, 1:64, 1:128 and higher. The results of the N tests have been divided in two groups: NI values of 3.5 or lower in one, 3.6 or higher in the other.

The observations on development of resistance to a challenge with EEE virus in guinea pigs shortly after vaccination can be summarized as follows.

Based on survival, statistically significant protection was apparent by the 3rd day after vaccination with the homologous vaccine; and, while only on the borderline of statistical significance, there was an indication that protection may appear 1 or 2 days after vaccination. Non-

specific protection was not observed when JE vaccine was given 24 hours before challenge.

The distribution of CF antibody titers in surviving guinea pigs presented a pattern which corresponded closely to that observed in terms of survival to the challenge: unvaccinated controls, with the highest mortality, had most CF titers in the high-titer class, 9 or 15; solidly protected guinea pigs vaccinated 7 days before challenge, with zero mortality, had most CF titers in the negative class, 12 of 15: In this they resembled the vaccinated unchallenged animals which had 5 of 7 in this category. Guinea pigs vaccinated from 1 to 4 days before challenge, which had shown following challenge mortalities of 12.5 and 31.6 percent, intermediate between controls--68.5 percent--and fully protected animals--0 percent--had also an intermediate position in the CF test results; the majority, 12 of 22, were in the 1:8 to 1:32 group. Finally, the guinea pigs vaccinated with JE vaccine which showed no protection (or at most the merest indication) had titers also in the intermediate group.

The results of the N tests showed that the NI could be placed in two sets; one with the higher values, included the sera from challenged unvaccinated controls, animals vaccinated with EEE vaccine 1 to 4 days before challenge and animals vaccinated with JE vaccine 24 hours before challenge; the second set with the lower values was made up of the sera from the fully protected guinea pigs vaccinated 7 days before challenge and those from vaccinated unchallenged animals.

Development of early resistance against JE virus in guinea pigs, after vaccination. Peripheral inoculation of available strains of JE virus to guinea pigs did not, as anticipated, cause death or visible signs of illness. In attempts to develop a vaccination-challenge model in this species, preliminary studies were made to establish certain practical parameters following inoculation of the virus by ip route: titration of viremia and development of CF and N antibodies.

After inoculation of a large dose of virus equivalent to 10^6 newborn mouse IC LD_{50}, Table 11, there followed a short period of viremia with the highest titers 24 hours after inoculation. It was not clear whether the virus recovered in the blood represented persisting virus or actual multiplication of the inoculum. No attempt has been made thus far to utilize viremia or its suppression to estimate resistance to a challenge; the use of this procedure in a group of 30 or 40 guinea pigs for several consecutive days is not easy, but it may be attempted later.

Investigations on the development of CF and N antibodies gave the results shown in Table 12.

As can be seen in the table, inoculation of JE virus over a dosage from 10^6 (dilution 10^{-4}) to 10^2 (dilution 10^{-8}) newborn mouse IC LD_{50} to guinea pigs resulted in a systematic, orderly development of CF antibodies; with the sera secured 21 days after inoculation (dilutions 10^{-5} to 10^{-8}), a sharp, clear titration of infectivity of the inoculum was obtained, which in the present example gave an IP ID_{50} (intraperitoneal infective dose 50) of $10^{-7.2}$. Assuming that challenge of vac-

cinated animals resulted in suppression of antibody development in a similarly systematic fashion, this model could be profitably used for the study of the problem at hand.

The N test, with undiluted serum and virus dilutions, gave an answer similar to that of the CF test in a much shorter time; all animals that in time became positive by CF test at 21 days, were already positive by N test at 10 days. The N test may, however, have disadvantages: the speed and intensity of the response may obliterate subtle quantitative differences, unless the serum-dilution method is used; and the labor and means needed far outweight those required for the CF test.

Vaccination and challenge. Having established that antibodies developed in a systematic fashion following inoculation of JE virus and as it appeared that a dilution 10^{-6} of mouse brain virus was effective but not excessive infective dose, $10^{1.2}$ IP ID_{50}, experiments were next carried out to see how vaccination would alter the response to a challenge measured by CF antibody development.

In the first of 2 experiments, Table 13, undiluted vaccine was given to a group of guinea pigs 7 days (#3) and to another 4 days (#4) before challenge; another group, #2, were vaccinated along with group #3 and not challenged; and another group, #1, were held as unvaccinated controls. All guinea pigs were bled, only once, 15 days after challenge, including group #2 which was not challenged. The result of the CF test with the sera is shown in Table 13.

The unvaccinated controls, group #1, had 2 animals with no detectable antibodies, presumed to have escaped infection; 6 of the remaining 7 animals had titers of 1:64 or 1:128. The vaccinated, unchallenged guinea pigs, group #2, responded to vaccination with titers usually in the middle range, 1:32, with one showing a titer of 1:64. Groups #3 and #4, vaccinated and challenged 7 and 4 days later respectively, responded almost exactly like group #2, given vaccine and no challenge. It appears, therefore, that challenge of unvaccinated guinea pigs resulted in a few instances in no response; in most cases infection occurred and resulted in CF titers of 1:64 and higher; whereas vaccinated and challenged animals responded as though they had been vaccinated only, i.e., they were protected.

The second experiment, Table 14, was similar to the first with the exception that the vaccine was used at dilution 1:10 and the guinea pigs were bled 14 and 24 days after challenge. It is clearly apparent that CF antibody titers of the several groups differed but little 14 days after challenge; on the later bleeding, however, the titers of the unvaccinated controls had increased; those of all the vaccinated animals whether challenged or not had, if anything, decreased with the result that in group 1, 5 of 8 guinea pigs had titers of 1:64 or higher, while in groups 2, 3, and 4 all animals had titers 1:16 and lower. The protective effect of the vaccine in groups 3 and 4 is evident; it seems, furthermore, that while antibodies attributable to vaccination had reached their peak on day 14 (relative to the challenge) the antibodies due to infection were not fully developed at that time.

Development of complement-fixing antibodies in guinea pigs following inoculation of several group B arboviruses. Exploratory efforts to employ development of CF antibodies in guinea pigs as a sign of infection following inoculation of group B viruses other than JE, have met with varied success; the results of these attempts are summarized in Table 15.

None of the viruses used caused death or apparent illness in any of the animals. Banzi virus was particularly effective; even after injection of as little as $10^{2.7}$ newborn mouse IC LD_{50} all guinea pigs had high antibody titers 21-22 days after inoculation; evidently this virus in guinea pigs gives an excellent model ot investigate the present problem.

The other viruses used were not very successful in inducing CF antibodies. Only 4 animals of 16 injected with yellow fever virus had antibodies 21 or 22 days later and only one had a reasonably high titer, 1:64. Inoculation of Powassan virus to 7 guinea pigs resulted in only one positive serum, 1:32, three weeks later. The low titers, 1:8 or 1:16, observed in 3 animals given the largest of the two doses of dengue 2 virus (dilution 10^{-2}) may have been a response to the mass inoculated rather than the result of virus multiplication; further studies are needed to settle this question, including tests on sera taken later than 15 days after inoculation.

VI. Discussion

In observations previously reported (Annual Report, May 1973) resistance to a challenge with VEE virus was detected in mice vaccinated 1 to 4 days earlier with the homologous virus, but not with a heterologous one; the challenge dose was 100 LD_{50} and the vaccine Colorado tick fever virus. In the current experiments an early resistance against a VEE virus challenge given 24 hours after vaccination with JE virus vaccine was noted; while the virus in the vaccine may have influenced the outcome, it appears more logical to assume that the smaller amount of virus in the challenge, 2.5 LD_{50}, may have been the principal reason for the early non-specific protection.

Yellow fever virus is of great importance in connection with the present project; no observations had been attempted in the past due to inability to challenge mice effectively by peripheral route. In spite of their artificiality, studies were made on the development of resistance to an ic challenge with the virus, which have revealed an interesting fact: effective protection was observed 24 hours—and later— after vaccination when the challenge was a strain, French viscerotropic, which had not been propagated in mice before, while no protection to speak of was observed when the challenge was a thoroughly mouse adapted strain, Asibi neurotropic. The determining factor may well have been that the viscerotropic strain was a slow acting virus in the mouse, while the Asibi strain was quick acting; conceivably, the viscerotropic strain being less rapidly invasive allowed for immunity due to the vaccine to overtake and block the lethal activity of the virus.

The effect of Cytoxan, given repeatedly at the time of vaccination and challenge, and using moderate to low doses of challenge virus, seems to have been characterized with the model used: it has failed to inhibit the early resistance to a challenge after vaccination. Since, in fact, Cytoxan increased the mortality of peripherally infected mice, the detection of early resistance was made easier. The current observations strengthened the prior conclusion that the early phase of protection following vaccination is not, or is not entirely, explicable on an immune mechanism.

Investigation of early resistance in guinea pigs is made difficult by the low, or lack of, susceptiblility in terms of lethality to some of the viruses that we had planned to study. The number of animals needed with a virus which kills only a proportion of controls, such as EEE, is much too large to use this species as a practical method to investigate small degrees of protection following vaccination. Other parameters, for example, titration of viremia following challenge of guinea pigs with JE virus, offered other possible ways of study. However, a simpler method appears to be the titration of antibodies in surviving challenged animals; studies with the CF test show a marked difference in the degree of response in variously treated groups of animals: vaccinated only, challenged only, and vaccinated and challenged at several days later.

Development of antibodies, or resistance to a second challenge, in experimental animals after a non-fatal infection is a method often used to detect infection. In the experiments reported the procedure has been applied and the results have been interpreted to indicate blocking of inapparent infection in animals which, judging by the response in unvaccinated controls should have developed antibodies. The method requires a well balanced combination of amount of vaccine, dose of challenge, and time after challenge when the survivors are bled; otherwise the differences between the responses of the several groups may be less apparent.

VII. Conclusions

1. Early resistance, i.e. resistance 24 hours after vaccination, against a small dose challenge with VEE virus was observed in mice vaccinated with VEE TC80 virus. A significant resistance against the same challenge was noted in mice vaccinated 24 hours earlier with JE vaccine, but not when this vaccine was given 7 days before challenge.

2. Vaccination of mice with live 17D strain of yellow fever resulted in early resistance, 1 to 4 days later, against an intracerebral challenge with yellow fever, French viscerotropic strain, but not against the Asibi neurotropic strain.

3. Administration of Cytoxan to mice immunized with arboviruses did not suppress or diminish early resistance to a homologous or heterologous challenge given 24 hours later.

4. Substantial resistance to a challenge with EEE virus was observed in guinea pigs vaccinated with homologous vaccine 3 or more days before challenge; a small degree of protection seemed apparent in animals vaccinated 1 or 2 days before challenge but its statistical significance was borderline.

5. Guinea pigs that survived a challenge with EEE or JE viruses developed high titered CF antibodies; animals that were vaccinated but not challenged developed low titered CF antibodies. Vaccinated and challenged guinea pigs developed antibodies with titers similar to those present in vaccinated only animals.

6. Titration of antibodies in vaccinated guinea pigs following a non-lethal challenge can be used as a method for detecting the efficacy of the vaccine.

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Table 1

Effect of Cytoxan on early phase resistance following vaccination of mice: vaccine JE virus, challenge EEE virus

Group No.	Regimen	Vaccine	Result of challenge, 1 day after vaccination, EEE virus dilution 10^{-5}	10^{-6}	10^{-7}
1	Cytoxan	JE	6/64		
2	Cytoxan	none	53/64	4/16	2/16
3	No Cytoxan	JE	1/64		
4	No Cytoxan	none	47/64	9/16	2/16

Mice dead/mice challenged

Table 2

Effect of Cytoxan on early phase resistance following vaccination in mice: vaccine EEE virus, challenge Banzi virus

Group No.	Regimen day 1	Regimen day 2	Regimen day 3	Result of challenge, Banzi virus, dilution 10^{-8}	10^{-9}	10^{-10}	LD_{50} controls	LD_{50} in dilution 10^{-8}	chi square at 10^{-8} vaccinated vs. controls
1	Cytoxan vaccine	challenge	Cytoxan	21/32					6.1
2	Cytoxan No vaccine	challenge	Cytoxan	30/32	9/14	1/16	$10^{-9.2}$	11	
3	No Cytoxan vaccine	challenge	No Cytoxan	13/32					5.7
4	No Cytoxan No vaccine	challenge	No Cytoxan	23/32	5/16	0/16	$10^{-8.5}$	3	

Mice dead/mice challenged

Table 3

Effect of large dosage of Cytoxan on early phase resistance following vaccination of mice: vaccine and challenge JE virus

Group No.	Regimen	vaccine	Result of challenge, 1 day after vaccination, JE virus dilutions 10^{-6}	10^{-7}	10^{-8}	chi square at 10^{-6}
1	Cytoxan, 5 injections	JE	14/28			15.5
2	Cytoxan, 5 injections	None	27/27	8/16	7/16	
3	No Cytoxan	JE	0/32	2/16	4/16	12.4
4	No Cytoxan	None	12/32			

Mice dead/mice challenged

Table 4

Vaccination of mice with live yellow fever (17D) virus and intracerebral challenge with yellow fever (Asibi) virus

Test no.	Vaccination, days before challenge	Challenge, dilution				
		10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
1	7	30/30	31/32			
	1	32/32	32/32			
	No vaccine	30/30	32/32	16/16	10/16	
2	7			24/39	7/40	
	1			36/40	10/40	
	No vaccine			38/40	17/40	2/16

Mice dead/mice challenged

Table 5

Titers and average survival times (AST) following intracerebral inoculation of yellow fever, strain Asibi and French viscerotropic, to mice 5 to 7 weeks old

Strain		Virus dilution							
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Asibi	Mortality				28/28	31/31	54/56	27/56	2/16
	AST				5.3	5.5	6.0	6.1	5.5
French visc.	Mortality	12/12	12/12	[illegible]/12	40/44	28/44	5/28	0/28	
	AST	8.9	9.7	10.4	10.4	12.1	14.7		

Mice dead/mice inoculated

AST, in days

Table 6

Vaccination of mice with live yellow fever (17D) virus and intracerebral challenge with yellow fever (French viscerotropic) Virus

Vaccination, days before challenge	Challenge, dilution			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
4	0/32	0/32		
3	2/31	0/32		
2	5/28	1/32		
1	3/32	1/30		
No vaccine	28/32	21/32	2/16	0/16

Mice dead / mice challenged.

Table 7

Early resistance following vaccination of mice against VEE Virus subcutaneous challenge

Vaccination		Challenge, dilution		
Vaccine	Days before challenge	10^{-9}	10^{-10}	10^{-11}
VEE	7	0/32		
	1	0/32		
JE	7	24/30		
	1	8/32		
No vaccine		26/32	1/15	0/16

Mice dead / mice challenged.

Table 8

Challenge of guinea pigs with EEE virus soon after vaccination with either EEE or JE viruses

Test No.	Vaccination: Vaccine	Vaccination: Days before challenge	Number of Animals	Result of challenge: Dead	Result of challenge: Survived	Result of challenge: % Mortality	Significant results, chi square
1	EEE	1	12	4	8	33	
		2	10	3	7	30	
	None		12	8	4	66	
2	EEE	1	16	5	11	31	
		7	16	0	16	0	9.06
	None		17	9	8	53	
3	EEE	3	16	1	15	6	10.6
		4	16	3	13	19	6.2
	None		16	11	5	69	
4	JE	1	26	16	10	61.5	
	None		25	20	5	80	

Table 9

Antibody development against EEE virus in surviving guinea pigs following vaccination and challenge with EEE virus

Vaccine	Days between vaccine and challenge	Complement-fixation Test							Neutralization Test			
		Number Tested	No. with indicated titer						Number Tested	Number with NI		
			<8	8	16	32	64	128		2.9 or <	3 - 3.5	3.6 or >
None	controls	15	1	1	1	3	3	6	15	2	2	11
EEE	no challenge	7	5	2					7	2	3	2
EEE	7	15	12	1	1		1		15	7	5	3
EEE	3 or 4	6	1		1	2		2	3			3
EEE	1 or 2	16	1		3	6	4	2	12	1	3	8
JE	1	8	1		1	4	2		8	1	1	6

Complement-fixation titers expressed as reciprocal of titer; 128, indicates 1:128 or higher.

NI, Neutralization Index given by undiluted serum.

Table 10

Summary of results with guinea pigs vaccinated and challenged with EEE virus: Protection and Antibodies

<table>
<tr><th rowspan="3">Vaccine</th><th rowspan="3">Challenge, days after vaccine</th><th colspan="4">Challenge</th><th colspan="3">CF Test</th><th colspan="2">N Test</th></tr>
<tr><th rowspan="2">Number of animals</th><th rowspan="2">Dead</th><th rowspan="2">sur-vived</th><th rowspan="2">% mor-tality</th><th colspan="3">Titer</th><th colspan="2">NI</th></tr>
<tr><th><8</th><th>8,16 or 32</th><th>64,128 or ></th><th>3.5 or <</th><th>3.6 or ></th></tr>
<tr><td>None</td><td>Controls</td><td>70</td><td>48</td><td>22</td><td>68.5</td><td>1</td><td>5</td><td>9</td><td>4</td><td>11</td></tr>
<tr><td>EEE</td><td>None</td><td>7</td><td>0</td><td>7</td><td>0</td><td>5</td><td>2</td><td>0</td><td>5</td><td>2</td></tr>
<tr><td>EEE</td><td>7</td><td>16</td><td>0</td><td>16</td><td>0</td><td>12</td><td>2</td><td>1</td><td>12</td><td>3</td></tr>
<tr><td>EEE</td><td>3 or 4</td><td>32</td><td>4</td><td>28</td><td>12.5</td><td rowspan="2">2</td><td rowspan="2">12</td><td rowspan="2">8</td><td rowspan="2">4</td><td rowspan="2">11</td></tr>
<tr><td>EEE</td><td>1 or 2</td><td>38</td><td>12</td><td>26</td><td>31.6</td></tr>
<tr><td>JE</td><td>1</td><td>26</td><td>16</td><td>10</td><td>61.5</td><td>1</td><td>5</td><td>2</td><td>2</td><td>6</td></tr>
</table>

CF Test: Reciprocal of titers.

NI, Neutralization Index of undiluted serum.

Table 11

Viremia in guinea pigs following intraperitoneal inoculation of Japanese encephalitis virus

Guinea pig	Days tested after inoculation and titer*			
	1	2	3	4
1		1.6		
2		1.2		
3		1.2		
4		0.6		
5	3.4	1.0	±	none
6	3.2	0.3	0.3	±
7	2.7	0.7	±	none
8	2.1	0.3	0.2	±

*Titer: $\log_{10}$; ±, 1 mouse died of 8 inoculated undiluted blood; none, no mice died of 8 inoculated.

The inoculum, 1 ml dilution 10^{-4}, contained approximately 10^6 IC LD_{50} by titration in newborn mice.

Table 12

Development of antibodies in guinea pigs following intraperitoneal inoculation of Japanese encephalitis virus

Number of Animals	Dilution inoculated ($ICLD_{50}$)	Day after inoculation when bled	CF test				N test			
4	$10^{-4}(10^{6})$	4	0	0	0	0	2.4	2.9	2.5	3.1
		11	8		32	32	3.8		2.5	3.1
		24	128		64	32	5.1+		5.1+	5.0+
4	$10^{-5}(10^{5})$	10	32	64	64	128				
		21	128	128	256	128	4.0+	3.9+	3.6+	4.0+
4	$10^{-6}(10^{4})$	10	0	0	0	0	3.0+	3.0+	2.8+	3.0+
		21	128	64	64	128	3.9+	3.9+	4.0+	4.0+
4	$10^{-7}(10^{3})$	10	0	64	0	0	0.4	2.5	0.3	2.8+
		21	0	128	0	128	<1.0	3.8+	<1.0	3.8+
4	$10^{-8}(10^{2})$	10	0	0	0	0	2.9	0.1	0.1	0.0
		21	128	0	0	0	4.0	<1.0	<1.0	<1.0

CF test: reciprocal of serum titer; 0, no fixation at dilution 1:8.

N test: neutralization index, intracerebral test in newborn mice.

Table 13

Development of complement-fixing antibodies in guinea pigs following vaccination and challenge with Japanese encephalitis virus

Group	Vaccine	Challenge, days after vaccine	Number of Animals	CF test, titer					
				<8	8	16	32	64	128
1	None	Yes, controls	9	2			1	2	4
2	Yes	None	8			1	6	1	
3	Yes	7	9			4	4	1	
4	Yes	4	9			3	5	1	

CF titer: reciprocal of titers.

Vaccine: 1 ml, undiluted.

Table 14

Development of complement-fixing antibodies in guinea pigs following vaccination and challenge with Japanese encephalitis virus

Group	Vaccine	Challenge, days after vaccine	Number of animals	Day bled after challenge	CF test, titer						
					<8	8	16	32	64	128	256
1	None	Yes	8	14		2	2	3		1	
				24			1	2	3	1	1
2	Yes	None	8	(14)		4	3	1			
				(24)		6	2				
3	Yes	7	8	14		6	2				
				24	1	4	3				
4	Yes	4	8	14		3	3	2			
				24		4	4				

CF test: reciprocal of titers.

Vaccine: 1 ml, diluted 1:10.

(14), (24): not challenged guinea pigs bled on the same days as those that had been challenged.

Table 15

Development of complement-fixing antibodies in guinea pigs following intraperitoneal injection of group B arboviruses

Virus	Dilution of virus inoculated (mouse IC LD_{50})	Number of animals	Day bled after inoculation and CF titers		
			10 or 11	15	21 or 22
Banzi	$10^{-6}(10^{4.7})$	4			128, 128, 256, 256
	$10^{-7}(10^{3.7})$	4			64, 128, 128, 128
	$10^{-8}(10^{2.7})$	4			128, 256, 256, 256
Dengue 2 (NGB)	$10^{-2}(10^{6.7})$	3		16, 16, 8	
	$10^{-4}(10^{4.7})$	3		0, 0, 0	
Powassan	$10^{-4}(10^{6.7})$	7	0, 0, 0, 0 0, 0, 0		0, 0, 0, 0 32, 0, 0
Yellow fever (French viscerotropic)	$10^{-4}(10^{5})$	4	0, 0, 0, 0		8, 4, 0, 0
	$10^{-5}(10^{4})$	4	0, 0, 0, 0		0, 0, 64, 0
	$10^{-6}(10^{3})$	4	0, 0, 0, 0		8, 0, 0, 0

CF titers: reciprocal of titer; 0, negative at dilution 1:4.